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13. ABSTRACT *(Maximum 200 Words)*

Women with germline mutations in the breast and ovarian cancer gene 1 (*Brca1*) have an approximately 50% lifetime risk of developing ovarian cancer and almost 90% chance of breast cancer. Brca1 mutations account for a significant percentage of all breast cancer cases. It appears that the main role for the Brca1 protein in cells is to prevent the accumulation of mutations in key growth regulatory genes in response to DNA damage. BRCA1 is phosphorylated in response to DNA damage by an elaborate surveillance mechanism, called a checkpoint, that detects DNA damage and prevents the accumulation of mutation. We are investigating the role these phosphorylation events play in the regulation of BRCA1. We have mapped phosphorylation sites and will mutate them to determine their function. We are also planning to investigate the mechanism through which the BRCA1 protein localizes to sites of DNA damage within cells.

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Introduction

Maintenance of genomic integrity is crucial for the development and health of organisms. Cell cycle checkpoints and DNA repair mechanisms help ensure the distribution of an intact genome to all cells and progeny. The inactivation of many of the genes involved in these activities have been linked to syndromes that cause a predisposition to cancer in humans. The *ATM*, *Brca1*, and *Brca2* genes are three such tumor suppressors involved in preventing genetic damage (1). Mutations in *ATM* cause ataxia telengiectasia (A-T), a disorder characterized by atrophy of the cerebellum and thymus, immunodeficiency, premature aging, predisposition to cancer, and sensitivity to ionizing radiation (2). Furthermore, heterozygous carriers of a dysfunctional ATM gene are predisposed to breast cancer (3). Mutations in *Brca1* and *Brca2* are linked to inherited, early-onset breast cancer (4). Mutations in *Brca1, Brca2*, or *ATM* cause defects in cellular proliferation, genomic instability, and sensitivity to DNA damage (5-7).

ATM is a member of a protein family related to phosphoinositide kinases that includes ATR, *MEC1*, *TEL1* and *RAD3*. These proteins are essential for signaling the presence of DNA damage and activating cell cycle checkpoints (8). ATM is activated in response to DNA damage and is required for efficient DNA double strand break repair and optimal phosphorylation and activation of the p53, c-Abl, and Chk2 proteins that promote apoptosis or cell cycle arrest (9-14).

The Brca1 and Brca2 proteins form a complex with Rad51, a RecA homologue required for homologous recombinational repair of DNA double stranded breaks (6,15-17). These three proteins localize to discrete nuclear foci during S phase of the cell cycle, share developmental expression patterns, and are maximally expressed at the G1-S transition (16-19). Brca1 mutations in mice result in genetic instability, defective G2/M checkpoint control and reduced homologous recombination (7). Exposure of cells to ionizing radiation or hydroxyurea causes dispersal of Brca1 foci and relocalization to sites

of DNA-synthesis where DNA repair may occur (18). Brca1 is phosphorylated during S-phase and is also phosphorylated in response to DNA damage (18,20).

Body

In the course of identifying BRCA1-associated proteins by mass spectrometry, we identified ATM and confirmed this association by reciprocal co-immunoprecipitation. Given this physical association, we tested whether ATM was required for phosphorylation of Brca1 in response to DNA damage. Brca1 from γ-irradiated wild-type cells migrated more slowly than the Brca1 from untreated cells on SDS-PAGE gels indicating phosphorylation (18, 20, and Fig. 1B). Brca1 in ATM-deficient fibroblast and lymphoblast cells derived from A-T patients was not hyper-phosphorylated after exposure to γ-irradiation (Fig. 1A). These studies were initiated prior to this grant and have been continued since then via this grant.

We confirmed that the lack of phosphorylation of Brca1 in A-T cells is dependent on the ATM-deficiency by examining A-T cells that had been complemented with an ATM cDNA. In the parental A-T cells or cells containing an empty vector there was only a slight shift of Brca1 protein after γ-
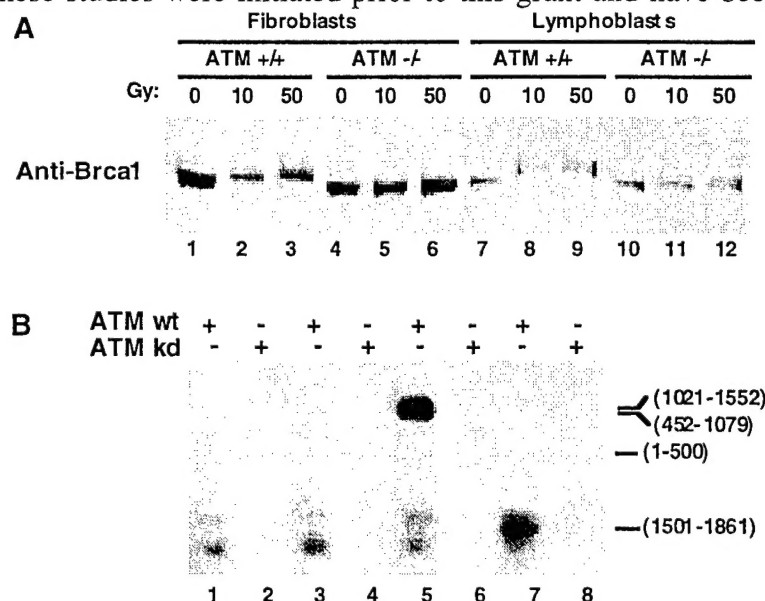
Figure 1. ATM-Dependent Brca1 Phosphorylation. (A) Wild type or A-T fibroblasts or lymphoblasts were treated with 10 or 50 Gy of γ-irradiation. Cell lysates were harvested one hour after irradiation, fractionated on SDS-PAGE, and immunoblotted with anti-Brca1 (Ab-1, Oncogene Research) antibody. (B) GST-fusion proteins containing Brca1 amino acids 1-500 (lanes 1 and 2), or 452-1079 (lanes 3 and 4), were used as substrates in an *in vitro* kinase assay with wild-type or kinase-defective ATM. The kinase reactions were separated by SDS-PAGE, stained with Coomassie blue and exposed to film. The two phosphorylated proteins observed in all of the odd numbered lanes are unidentified proteins co-immunoprecipitated with ATM from 293T cells.

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irradiation (Data not shown), but addition of the ATM expression vector increased the shift. Therefore, functional ATM is required for maximal γ-irradiation-induced phosphorylation of Brca1. In contrast, Brca2 is not required for γ-irradiation-induced phosphorylation of Brca1 (Data not shown)).

To determine whether ATM could phosphorylate Brca1, we produced several overlapping fragments of Brca1 fused to glutathione S-transferase (GST) in *E. coli* and used these as substrates in an ATM-protein kinase assay. Fusion proteins containing Brca1 amino acids 1021 to 1552 and 1501 to 1861 were phosphorylated by wild-type but not a catalytically-inactive mutant of ATM (Fig. 1B). Most phosphorylation occurred between amino acids 1351-1552. Brca1 segments containing amino acids 1021 to 1211 and 1211 to 1351 were also phosphorylated to a lesser degree suggesting that more than one residue may be targeted.

Analysis of the Brca1 sequence within this region revealed a cluster of serines and threonines followed by glutamine (SQ or TQ). SQ or TQ sequences are the preferred sites of ATM phosphorylation on p53 and c-Abl (9,10). The 244 amino acids between 1280 and 1524 contain 10 SQ/TQ sites whereas the remaining 1619 amino acids of Brca1 contain only 8 SQ/TQ sites.

We mapped the in vitro ATM phosphorylation sites on GST-Brca1 1021-1552 by mass spectrometry. Two phosphopeptides were identified by analysing proteins before and after treatment with calf-intestinal phosphatase (CIP). Peptide sequencing revealed that S1423 and S1524 were phosphorylated (Table 1). Six additional serines can be phosphorylated within amino acids 1021-1552 when the kinase reaction is allowed to proceed longer with higher concentrations of ATP and ATM (Table 1).

Table 1. Summary of in vitro and in vivo phosphorylated sites on Brca1 as detected by mass spectrometry.

Site	Peptide
In vitro	
S1330	^{326}HQSE**S**QGVGLSDKELVSDDEER^{1347}
S1423	^{1407}LQQEMAELEAVLEQHG**S**QPSNSYPSIISDSSALEDLRNPEQSTSEK^{145}
S1466	^{1460}SSEYPI**S**QNPEGLSADKFEVSADSSTSK^{1487}
S1524	^{1521}NYP**S**QEELIKVVDVEEQQLEESGPHDLTETSY^{1552}
S1542	^{1531}VVDVEEQQLEE**S**GPHDLTETSY^{1552}
?	^{1279}ASQEHHLSEETKCSASLFSSQCSELEDLTANTNTQDPFLIGSSK^{1322}
In vivo	
S1189	^{1189}**S**PSPFTHTHLAQGYR^{1203}
S1457	^{1453}AVLT**S**QKSSEYPISQNPEGLSADKFEVSADSSTSK^{1487}
S1542	^{1531}VVDVEEQQLEE**S**GPHDLTETSYLPR^{1555}
S1524, S1542	^{1521}NYP**S**QEELIKVVDVEEQQLEE**S**GPHDLTETSYLPR^{1555}

[#]Amino acids in underlined bold faced type were unambiguously determined to be sites of phosphorylation by LC/MS/MS. [*]At least one phosphorylated amino acid within these peptides could not be determined unambiguously by LC/MS/MS. [†]Peptides with both one and two moles of phosphate were observed.

To determine if the ATM-dependent phosphorylation of S1423 or S1524 also occurred in vivo, we expressed Brca1 amino acids 1351-1552 fused to the flag epitope and the SV40 large T-antigen nuclear localization signal (NLS). This flag-NLS-tagged Brca1 segment localized to the nucleus of transfected cells. Protein immuno-blotting showed that this segment migrated as several distinct bands on SDS-PAGE (Data not shown). This may reflect phosphorylation on multiple sites given that the CDK2 phosphorylation site on Brca1 also maps to this domain (21). The mobility of a proportion of this protein was reduced following exposure of these cells to γ-irradiation. Co-expression of wild-type ATM but not catalytically-inactive ATM increased the number and intensity of slower migrating bands observed after γ-irradiation. Phosphatase treatment increased the

mobility of the shifted proteins demonstrating that the altered migration does reflect phosphorylation. The 1351-1552 Brca1 fragment was also phosphorylated after γ-irradiation in wild-type but not ATM-deficient fibroblasts, further supporting the hypothesis that this Brca1 fragment contains ATM-dependent, in vivo phosphorylation sites.

We introduced S1423A and S1524A mutations in Brca1 1351-1552. Phosphorylation of the mutant Flag-NLS-tagged protein was reduced compared to that of the wild-type protein (Fig. 2A). Phosphorylation in the absence of DNA damage was also reduced by expression of catalytically-inactive ATM or by the S1423 and S1524 mutations. The mutated Brca1 segment was shifted slightly in response to γ-irradiation but this effect was not increased by wild-type ATM expression (not shown).

To determine the functional significance of ATM-dependent phosphorylation of Brca1 we attempted to complement the radiation hypersensitivity of Brca1-deficient cells with wild-type or mutant Brca1 proteins. We produced recombinant retrovirus encoding HA-tagged, full-length, wild-type Brca1 and a S1423A-S1524A mutant (SQ2). We infected the Brca1-mutant HCC1937 cell line, and selected for stable, polyclonal cell populations. Analysis of the expression of exogenous Brca1 in these cells by protein immuno-blotting with antibodies to HA revealed equal expression of the mutant and wild type proteins (Fig. 2A). HCC1937 cells have a truncated Brca1 protein expressed at low levels compared to wild-type cells. Immunoblotting with antibodies specific to Brca1 revealed that the expression of the exogenous HA-tagged Brca1 proteins was similar to that of the mutant endogenous protein (22). Expression of large amounts of Brca1 from heterologous promoters is not expected because it causes growth inhibition (23,24). Both the SQ2 mutant and wild-type Brca1 localized to discrete nuclear foci (Fig. 2B and C).

We examined the sensitivity to γ-irradiation of these cells by counting viable cells 3 days after damage. Little cell death occurred during this time period in any of the cell populations. Thus differences in cell number are mainly caused by differences in the

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ability of the cells to recover and proliferate after the damage. Therefore, damage-induced inhibition of cell proliferation served as a measure of the sensitivity of these cells to irradiation. Expression of wild-type Brca1, but not the SQ2 Brca1 mutant, significantly decreased the growth inhibition caused by 1 Gy of irradiation compared to that in cells expressing an empty vector (ANOVA, p=.0006) (Fig. 2D). Brca1 afforded less protection after 2 Gy of irradiation, but its effect was still significantly different from that of the SQ2 mutant. Doses greater than 2 Gy resulted in essentially no recovery or proliferation of any of the cell populations within the 3 days of this experiment (22).

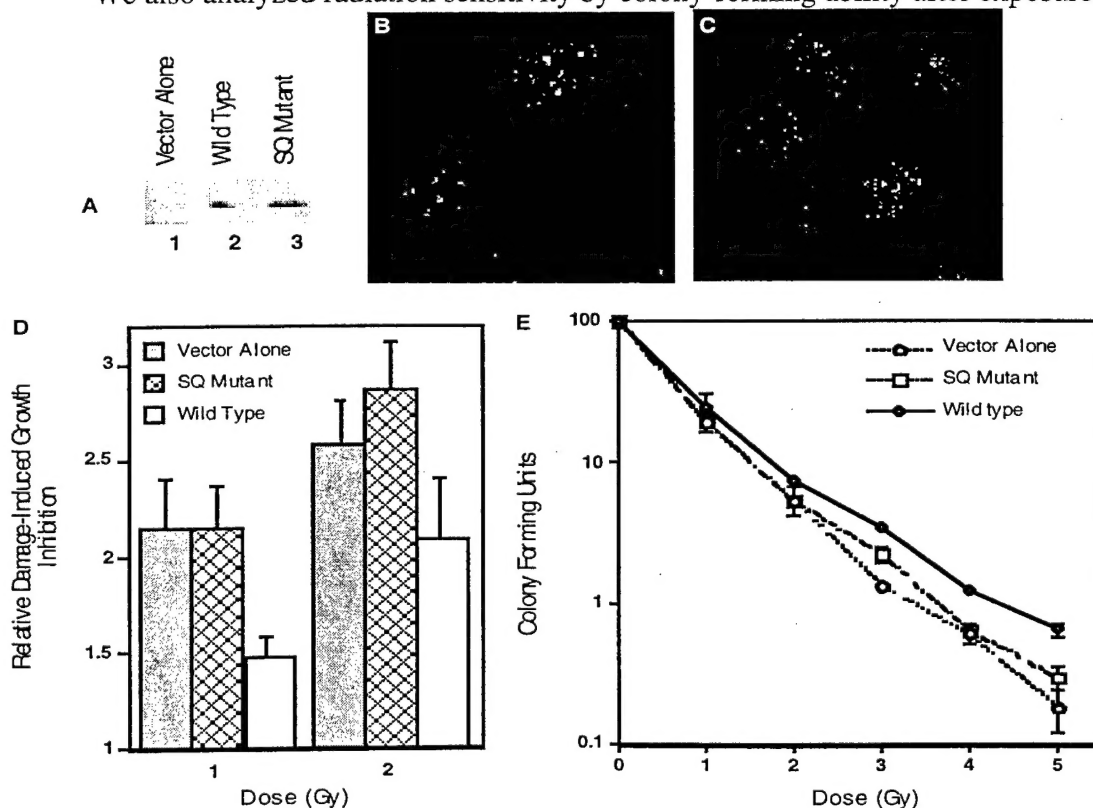We also analyzed radiation sensitivity by colony-forming ability after exposure of

Figure 2. Expression of wild-type Brca1 but not a phosphorylation mutant decreases the sensitivity of Brca1-deficient cells to ionizing radiation. Recombinant retrovirus expressing full length, HA-tagged wild type Brca1 or S1423A/S1524A Brca1 (SQ mutant) was used to create stable, polyclonal populations of HCC1937 cells (Brca1-deficient breast cancer cell line). Expression of the full length proteins was detected by western blotting (A) or immunofluorescent staining with anti-HA antibodies (panel B is wild-type Brca1, and panel C is the S1423A,S1524A mutant). (D) Cell populations were treated with the indicated doses of γ-irradiation, and viable cells were counted by trypan-blue exclusion 3 days after irradiation (Both floating and adherant cells were collected). Percent growth inhibition was calculated by dividing the number of cells in the treated population into the number of cells from the untreated population. By definition, the untreated samples have a growth inhibition of 1. (E) Cells were stained with methylene blue 13 days after γ-irradiation with the indicated doses, and the number of colonies was scored. Cell clusters of 10 or greater cells were scored as colonies. Numbers were normalized by setting the untreated sample at 100%.

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cells to various doses of γ–irradiation. Expression of wild-type Brca1, but not the SQ2 mutant, produced a significant decrease in the sensitivity of HCC1937 cells to γ–irradiation (Fig. 2E). Thus, phosphorylation of Brca1 on serines 1423 and 1524 is important for Brca1 function after exposure to γ–irradiation.

The mobility of full length Brca1 containing the S1423A and S1524A mutations on SDS-PAGE is still retarded after treatment with γ–irradiation (22) indicating that other residues besides S1423 and S1524 may also be targets for damage-induced phosphorylation. To examine this, we mapped damage-induced phosphorylation sites on Brca1 in vivo using mass spectrometry. Following irradiation of 293T cells that had been co-transfected with expression vectors for full length Brca1 and ATM, Brca1 was phosphorylated on five serines (Table 1). Four of these sites are in the SQ domain and three match sites phosphorylated by ATM in vitro. In addition, treatment of cells with HU appeared to give the same pattern of phosphorylation as described in technical objective 2. Since it does not appear that new sites are being phosphorylated in response to HU, we will continue to make the mutants from the ATM phosphorylation to understand the significance of BRCA1 phosphorylation. We anticipate that these alleles will appear to be defective in some aspect of BRCA1 response to replication blocks as well as to ionizing radiation.

Key Research Accomplishments

A) Development of an assay system to detect the function of BRCA1 mutants using HCC 1937 cells.

B) Identification of six sites of phosphorylation on BRCA1 by checkpoint kinases.

Reportable Outcomes

None yet to report.

Conclusions

As yet it is unclear how ATM-dependent phosphorylation regulates Brca1 function. We have not observed any differences in Brca1 localization in wild-type or A-T cells after exposure to γ–irradation (26). Thus, ATM may not be essential for regulating the intracellular localization of Brca1. Phosphorylation may regulate the binding of other proteins to Brca1. The Brca1 binding domain for Brca2 has been mapped to a region containing the SQ cluster; however, damage-dependent phosphorylation has not been shown to alter the amount of Brca2 complexed with Brca1 (17). The activity of Brca1 as a transcriptional regulator may be changed by phosphorylation since Brca1 has a transcriptional activation domain that maps to just downstream of the SQ cluster domain (27). Alternatively, the activity of Brca1 in modulating DNA repair may be altered by phosphorylation. Since DNA repair is defective in both ATM and Brca1-deficient cells it is tempting to speculate that the defect in repair and genetic instability found in ATM-deficient cells is at least partially caused by an inability to properly regulate Brca1.

The biochemical link between ATM and Brca1 may partially explain why heterozygous carriers of a dysfunctional ATM gene are at increased risk of breast cancer (3). It has been estimated that 6.6% of all breast cancer cases occur in the 1.4% of women who are A-T heterozygotes. Combined with the approximately 1 to 3% of breast cancers attributable to inherited Brca1 mutations, mutation of the ATM-Brca1 checkpoint/DNA repair pathway may account for nearly 10% of all breast cancer cases. Mice heterozygous for ATM mutations are more sensitive to sub-lethal doses of ionizing radiation than wild-type mice (28). Given the specific defect of ATM mutants in regulation of Brca1 in response to ionizing radiation, these results may have relevance to the issue of the relative

benefits of broad X-ray-based mammography screening for the early detection of breast cancer, a question to be resolved by epidemiological studies.

It is clear that Brca1 tumor suppressor protein is phosphorylated in response to DNA damage. The checkpoint protein kinase ATM was required for phosphorylation of Brca1 in response to ionizing radiation but not HU. ATM resides in a complex with Brca1 and phosphorylated Brca1 in vivo and in vitro in a region that contains clusters of serine-glutamine residues. Phosphorylation of this domain appeared to be functionally important because a mutated Brca1 protein lacking two phosphorylation sites failed to rescue the radiation hypersensitivity of a Brca1-deficient cell line. The same sites appear to be phosphorylated in response to HU. Thus, phosphorylation of Brca1 by the checkpoint kinase ATM and probably ATR may be critical for proper responses to DNA double strand breaks and replication stress and may help provide a molecular explanation for the role of ATM in breast cancer. We hope that the final analyses of these mutant can be carried out in the next period of this grant as well as the characterization of the localization of BRCA1 into foci. We are hoping to find genes that may be involved in controlling the foci formation of BRCA1.

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